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Fast protein motions are coupled to enzyme H-transfer reactions.

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KEYWORDS: *Kinetic isotope effects, temperature dependence, quantum tunneling, vibrational motion, promoting motions.*

ABSTRACT: Coupling of fast protein dynamics to enzyme chemistry is controversial and has ignited considerable debate, especially over the last 15 years in relation to enzyme-catalysed H-transfer. H-transfer can occur by quantum tunneling and the temperature-dependence of kinetic isotope effects (KIEs) has emerged as the ‘gold standard’ descriptor of these reactions. The anomalous temperature dependence of KIEs is often rationalised by invoking fast motions to facilitate H-transfer, yet crucially, direct evidence for coupled motions is lacking. The fast motions hypothesis underpinning the temperature dependence of KIEs is based on inference. Here, we have perturbed vibrational motions in pentaerythritol tetranitrate reductase (PETNR) by isotopic substitution where all non-exchangeable atoms were replaced with the corresponding heavy isotope (¹³C, ¹⁵N and ²H). The KIE temperature dependence is perturbed by heavy isotope labeling, demonstrating a direct link between (promoting) vibrations in the protein and the observed KIE. Further we show that temperature independent KIEs do not necessarily rule out a role for fast dynamics coupled to reaction chemistry. We show causality between fast motions and enzyme chemistry and demonstrate how this impacts on experimental KIEs for enzyme reactions.

INTRODUCTION

The potential involvement of fast (femtosecond to picosecond) dynamics in enzyme-catalysed reactions has been the focus of considerable debate, particularly in relation to modulation of the reaction free energy barrier¹⁻⁴. Experimentally it has been difficult to provide compelling evidence for any relationship between fast motions (as opposed to e.g. slower ms-ns loop opening/closing which are described elsewhere⁵) and the reaction chemistry. The importance of fast motions has been largely inferred from computational atomistic simulations of enzyme systems⁶⁻¹², or more qualitatively from experimental studies of reactions rates (reviewed in refs¹³⁻¹⁶), sometimes combined with numerical modelling using simple models of catalysis¹⁷⁻²¹. Debates concerning the notional coupling of fast dynamics to the reaction coordinate have, in the main (but not exclusively²²⁻²⁵), emerged from studies of enzymatic H-transfer involving quantum mechanical tunnelling (QMT). While most studies have employed the temperature²⁶ and more recently pressure^{17, 27} dependence of kinetic isotope effects (KIEs), in selected cases, indirect spectroscopic probes have also supported a role for fast dynamics in H-transfer reactions²⁸⁻³⁰.

Since the late 1990s, there have been many observations of unusual temperature dependencies of primary KIEs for enzymatic H-transfers, demonstrating that the occurrence of QMT is widespread. Measurement of the temperature-dependence of KIEs (ΔE_a) and associated values for Arrhenius prefactor ratios ($A_H:A_D$) has become the ‘gold standard’ for investigating enzymatic H-tunnelling and dynamics^{13, 26}. This follows as, unlike with small molecule systems³¹ and natural light-activated biological catalysts³²⁻³⁴, enzyme catalysts are not

amenable to studies at cryogenic temperatures or across the wide temperature ranges typically used to study tunnelling phenomena. Modelling KIE temperature dependence using simple vibronic models has given some limited, semi-quantitative insight into tunnelling and the inferred importance of motions coupled to the reaction coordinate^{10, 19-21, 35}. However, these models^{18, 20, 36, 37} have attracted criticism^{3, 38}. For example, they do not accommodate the multidimensional nature of QMT and they assume that room temperature tunnelling occurs nonadiabatically, which is inappropriate for most hydride and proton transfers. In vibronic models, the temperature dependence of the KIE is inferred from fast ‘distance sampling’ as the reactant state approaches a tunnelling-ready configuration^{20, 26}. Analogous distance sampling mechanisms could, in principle, enhance the rate of a classical transfer reaction by modulating the properties of the free energy barrier.⁴ The temperature dependence of reaction rates for QMT in enzymes reports on the need to achieve a conformation of the enzyme-substrate complex in which donor and acceptor wells for H or ²H (D) transfer are degenerate to facilitate the tunnelling reaction.²⁶ The contentious aspect is why this temperature dependence is sometimes different (and in other cases not) for H and ²H transfer, giving rise to temperature (in)dependent KIEs. Given that this is the primary descriptor used to identify tunnelling behaviour, it is crucial to understand if thermally accessible fast motions (i.e. vibrational coupling of fast enzyme motions to the reaction coordinate) influence temperature dependence of the KIE. The interpretation of the temperature dependence of KIEs can be addressed through atomistic simulation of tunnelling and dynamics including combined quantum mechanics/molecular mechanics (QM/MM) methods,

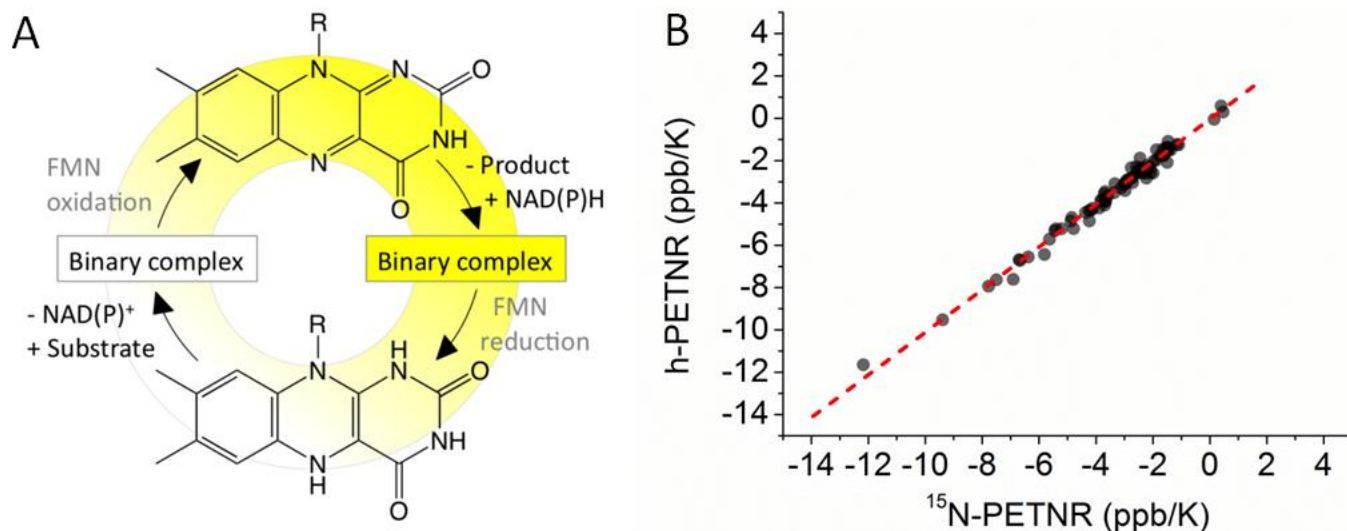


Figure 1. Catalytic cycle of PETNR and NMR evidence for the isostructural nature of l-PETNR and h-PETNR. **a**, PETNR catalytic cycle. In the reductive half-reaction, hydride transfer proceeds from the C4 *pro-R* hydrogen of NADH to the N5 atom of FMN resulting in a bleaching of the of the FMN cofactor absorption. This bleaching of absorption reports on the hydride transfer chemistry from NAD(P)H. The oxidative half reaction may proceed with molecular oxygen or with a series of oxidising organic substrates^{56, 57}. **b**, Correlation of NMR temperature coefficients for h-PETNR and ¹⁵N-PETNR residues. Dashed line is a diagonal intersecting zero. See *Materials and Methods* for experimental conditions.

variational transition state theory (with ensemble averaging and multidimensional tunnelling calculations),³⁹ spectral density/molecular dynamics simulations⁶ and empirical valence-bond⁴⁰/combined quantum classical path centroid path integral approaches⁴¹. Direct experimental probes of the origin(s) of the temperature dependence of KIEs are more difficult.

Recently, Silva and co-workers provided important insight into the coupling of fast motions to barrier crossing in the classical reaction catalysed by human purine nucleoside phosphorylase (PNP)²³. The kinetics of arsenolysis by PNP and a ‘heavy’ version of the enzyme, where ¹²C, ¹⁴N and non-exchangeable ¹H atoms were replaced with the corresponding isotope (¹³C, ¹⁵N and ²H; total mass increase of 9.9 %), were investigated. The Born-Oppenheimer approximation was invoked to infer that the potential energy surface for nuclear motion was unaltered in the different enzyme forms, but that the frequencies of protein vibrational modes were decreased. The rates of arsenolysis of inosine and guanosine decreased by 15 % and 27 %, respectively, with heavy PNP, and a similar decrease in the forward commitment to catalysis was observed. These data are consistent with the coupling of some vibrational mode(s) to the chemical coordinate during barrier crossing through the classical transition state, which the authors infer reflect fast dynamics.

We have now adapted the approach of Silva and co-workers to investigate the impact, or otherwise, of fast motions on the temperature dependence of KIEs in enzymatic QMT reactions. Hydride transfer from the nicotinamide coenzymes NADH or NADPH catalysed by the flavin mononucleotide (FMN)-dependent pentaerythritol tetranitrate reductase (PETNR) occurs, in the main, by QMT and this chemical step in the reaction cycle (Figure 1A) is readily accessed using stopped-flow methods with conventional and deuterated coenzyme⁴². Previous stopped-flow studies indicate that KIEs for FMN reduction are temperature-dependent with NADPH ($\Delta E_a = 6.5 \pm 2.8$ kJ mol⁻¹) and, within experimental error, temperature-independent with NADH ($\Delta E_a = -1.1 \pm 2.1$ kJ mol⁻¹)⁴², imply-

ing a stronger coupling of fast motions with the reaction coordinate with NADPH (if indeed the origin of temperature-dependent KIEs is fast motions). This inference is also consistent with studies of the pressure dependence of the KIEs⁴². The varied response of PETNR in relation to the temperature dependence of KIEs with NAD(P)H/²H enables a definitive test of the fast coupled motions hypothesis to be conducted. Here we report the temperature-dependence of the KIE with ‘heavy’ forms of PETNR using both NADH and NADPH coenzymes. We show that the temperature-dependence of primary KIEs reports on the coupling of protein vibrational modes to the reaction coordinate in this QMT reaction. Fast motions in enzymes are therefore coupled to the chemical (H-transfer) step and their perturbation (by stable isotope labelling) demonstrates a causal effect on the H-transfer rate by modulation of the reaction free energy barrier.

Materials and Methods

Enzyme and coenzyme preparation. l-PETNR was expressed and purified as described previously.⁴³ Isotopically labelled PETNR was prepared by growth of the expression system in minimal media (M9) substituted with labelled H, N and C sources (¹⁵NH₄Cl for ¹⁵N-PETNR, and ¹⁵NH₄Cl, ²H₇-¹³C₆ and ²H₂O for h-PETNR) essentially as described previously⁴⁴. Expression and purification protocols were essentially identical to l-PENTNR. (R)-[4-²H]-NADH and (R)-[4-²H]-NADPH were prepared as described previously⁴². Accurate mass determination was performed by the Manchester Biomolecular Analysis Facility on an Agilent 6510 Q-Tof.

NMR. Isotopically enriched ¹⁵N,¹³C,²H-PETNR and ¹⁵N-PETNR were exchanged into 50 mM potassium phosphate buffer pH 7.0 and concentrated to 0.5 mM using 10 kDa molecular weight cut-off Vivaspin concentrators (GE Healthcare). The samples for NMR analysis (280 μ l) were supplemented with 1 mM NaN₃, 10% v/v D₂O and 0.2 mM sodium 3-(trimethylsilyl)propionate-2,2,3,3,-d₄ (TSP) and introduced into 5-mm Shigemi tubes. ¹⁵N-TROSY experi-

ments (spectral width: ^1H = 20 ppm, ^{15}N = 34 ppm (offset = 118 ppm);

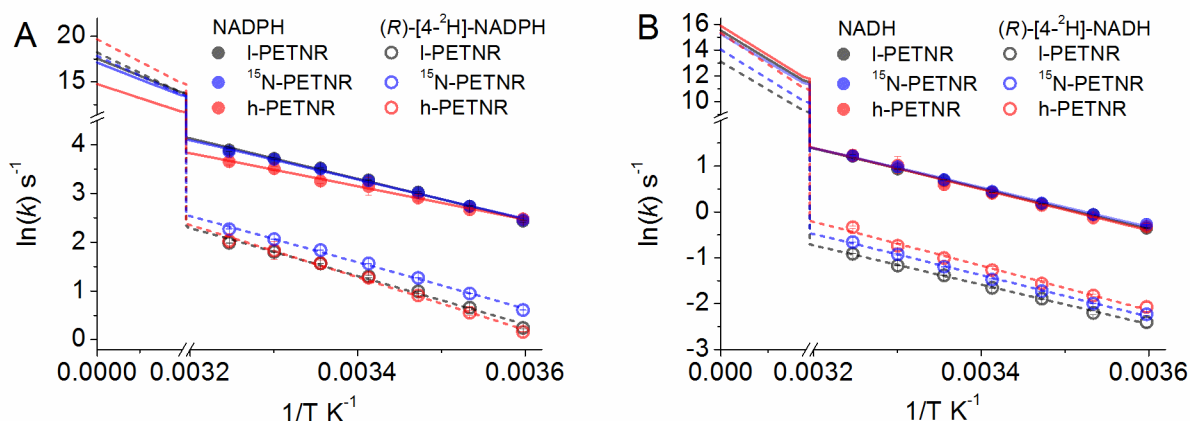


Figure 2. Temperature dependence of KIEs from stopped-flow studies. Temperature-dependence of FMN reduction in I-, ^{15}N - and h-PETNR with (A) NADPH and (B) NADH. Solid lines show fits to the Arrhenius equation. Ordinate intercepts of fitted lines show the (log) pre-factor values. Solid circles and lines are for H-transfer. Open circles and dashed lines are for D-transfer. See Table 1 for extracted parameters. Conditions, 50 mM potassium phosphate pH 7, 10 μM enzyme and 5 mM (NADPH) or 25 mM (NADH) coenzyme.

complex data pairs: ^1H = 1024, ^{15}N = 128; acquisition time: ^1H = 85.2 ms, ^{15}N = 61.9 ms) were acquired at 278, 288, 295 and 308 K for ^{15}N , ^{13}C , ^2H -PETNR and ^{15}N -PETNR using a 600 MHz Bruker Avance spectrometer equipped with a TXI cryoprobe and triple-axis gradients. Temperature calibration of the spectrometer was determined using d_4 -methanol. Spectra were processed and analysed using FELIX 2007 (Felix NMR, Inc., San Diego, CA) and the temperature dependences of the backbone HN chemical shifts were obtained using least-squares linear fitting. Proton chemical shifts were referenced to the methyl signals of TSP at 0.0 ppm and ^{15}N chemical shifts were calculated indirectly using the gyromagnetic ratio $^{15}\text{N}/^1\text{H}$ = 0.101329118.

Stopped-flow kinetics. All experiments were performed in 50 mM potassium phosphate, pH 7. To prevent oxidase activity of PETNR, all kinetic studies were performed under strict anaerobic conditions within a glove box (Belle Technology; <5 ppm O_2) using a Hi-Tech Scientific (TgK Scientific, Bradford on Avon, U.K.) stopped-flow spectrophotometer housed inside the glove box. Spectral changes accompanying FMN reduction were monitored at 465 nm using a saturating concentration of NADH/(R)-[4- ^2H]-NADH (25 mM) or NADPH/(R)-[4- ^2H]-NADPH (5mM), prepared as described previously.⁴⁵ Typically 3 - 5 measurements were taken for each reaction condition. Reaction transients were fit using a single exponential function.

Results and Discussion

Protein structure and reactive geometries in ‘light’ and ‘heavy’ PETNR.

Unlabelled (natural abundance) PETNR (light-PETNR [I-PETNR]) was isotopically enriched with ^{13}C , ^{15}N and ^2H (to give heavy-PETNR [h-PETNR]) or ^{15}N alone (to give ^{15}N -PETNR) giving a total mass increase assessed by mass spectral analysis of 9.8 % and 1.3 %, respectively. Isotopic substitution is not known to alter the structure of proteins. However, our experimental system relies on only the frequency of protein vibrational modes changing on isotopic substitution, so the similarity of the structures of h- and ^{15}N -PETNR was established using NMR spectroscopy. A direct comparison of

chemical shifts is inadequate as individual nuclei are affected by isotope shifts, whose magnitudes are varied⁴⁶. Hence, the temperature dependences of the backbone HN chemical shifts (the temperature coefficients⁴⁷) were used as highly sensitive probes of structure perturbation. Figure 1B shows the correlation of temperature coefficients of each PETNR residue for both h-PETNR and ^{15}N -PETNR. The data are well described by a diagonal intersecting zero (Figure 1B; dashed line), demonstrating that h-PETNR and ^{15}N -PETNR (and by inference I-PETNR) are isostructural.

The geometry of the reactive complex can be probed very precisely by monitoring the charge transfer (CT) complex absorbance band that arises when NAD(P)H binds to PETNR and related enzymes^{19, 48}. This is achieved using non-reactive mimics of NAD(P)H [1,4,5,6-tetrahydro-NAD(P)H, NAD(P)H₄], which form a stable binary complex with PETNR, accurately mirroring the reactive complex with NAD(P)H. This CT absorbance band at 555 nm acts as a ‘spectroscopic ruler’ for variation in the donor-acceptor (nicotinamide C4 to FMN N5) distance (Figure S1)¹⁹. Figure S2 shows the concentration dependence of CT absorbance with increasing NAD(P)H₄, with the resulting fitting parameters given in Table S1. The values of the coenzyme-saturated CT absorbance and the PETNR-NAD(P)H₄ complex dissociation constants do not significantly differ between I-PETNR and h-PETNR with either NADPH₄ or NADH₄. These data provide compelling evidence that the reactive geometries (binary complexes) of I-PETNR and h-PETNR are indistinguishable for both coenzymes.

A continuum of temperature-dependent KIEs

The temperature dependence of the observed rate constants and primary H^2/H KIEs for the reactions with both NADH and NADPH (either protiated or specifically deuterated at the pro-R C4-H to make (R)-[4- ^2H]-NAD(P)H) was measured with I-PETNR,⁴² ^{15}N -PETNR and h-PETNR (Figure 2 and Figure S3; Table 1; Table S2-S4). Note that the comparison of the h-PETNR and ^{15}N -PETNR data allows any isotope effect specifically arising from ^{15}N -labeling of the FMN cofactor (specifically the acceptor N5; Figure 1A) to be accounted for. As was

observed previously for l-PETNR⁴², the reaction proceeds ~1 order of magnitude faster at room temperature with NADPH than with NADH, and the magnitude of both KIEs at this temperature is ~7 (Table S5). As the temperature dependencies of these reactions all vary, comparison of the magnitude

Table 1. Extracted temperature dependence parameters from Figure 2.

| | l-PETNR ^c | ¹⁵ N-PETNR | h-PETNR |
|--------------------|-----------------------------|-----------------------------|-----------------------------|
| NADPH | | | |
| E_{aH}^a | 34.8 ± 1.0 | 33.7 ± 0.9 | 28.3 ± 0.9 |
| E_{aD}^a | 38.6 ± 1.3 | 37.2 ± 1.1 | 44.9 ± 1.6 |
| $\Delta E_a^{a,b}$ | 6.5 ± 2.7 | 5.9 ± 2.0 | 16.6 ± 2.4 |
| A_H | $(4.1 \pm 1.7) \times 10^7$ | $(2.6 \pm 1.0) \times 10^7$ | $(2.5 \pm 0.9) \times 10^6$ |
| A_D | $(7.9 \pm 5.6) \times 10^7$ | $(5.4 \pm 2.4) \times 10^7$ | $(3.4 \pm 2.2) \times 10^8$ |
| $A_H:A_D$ | 0.5 ± 0.15 | 0.48 ± 0.28 | 0.01 ± 0.01 |
| NADH | | | |
| E_{aH}^a | 36.7 ± 0.6 | 36.0 ± 0.9 | 37.6 ± 1.9 |
| E_{aD}^a | 35.9 ± 0.7 | 37.7 ± 0.8 | 40.3 ± 1.7 |
| ΔE_a^a | -0.8 ± 1.3 | 1.7 ± 1.7 | 2.7 ± 3.6 |
| A_H^b | $(5.4 \pm 1.3) \times 10^6$ | $(4.1 \pm 1.6) \times 10^6$ | $(7.8 \pm 6.2) \times 10^6$ |
| A_D | $(4.8 \pm 1.3) \times 10^5$ | $(1.2 \pm 0.4) \times 10^6$ | $(4.5 \pm 3.2) \times 10^6$ |
| $A_H:A_D$ | 11.3 ± 4.3 | 3.3 ± 1.7 | 1.7 ± 1.9 |

Parameters determined from fits of the data in Figure 2 to the Arrhenius equation. a, kJ mol⁻¹. b, $\Delta E_a = E_{aD} - E_{aH}$. c, reported in ref 42.

of the observed rate constants and KIEs at a reference temperature is not meaningful. The majority of the variation in the temperature dependencies of the KIEs arises from reactions involving deuterium (rather than protium) transfer (Figure 2; Table 1). Klinman and co-workers have similarly observed changes in temperature-dependence data arising predominantly from ²H-transfer⁴⁹, suggesting that such differences arise due to the stronger dependence of ²H-transfer on the donor-acceptor tunneling distance (in PETNR the nicotinamide C4 to FMN N5 distance; Figure 1A). That is, the longer wavelength of protium means that H transfer is less sensitive to donor-acceptor distance fluctuation than ²H transfer.

The l-PETNR, ¹⁵N-PETNR and h-PETNR behaved similarly, with temperature-dependent KIEs observed on the reactions with NADPH ($\Delta E_a > 0$; $A_H:A_D < 1$)²⁶ and apparently temperature-independent KIEs with NADH ($\Delta E_a \sim 0$; $A_H:A_D > 1$) (Table 1). Figure 3 indicates the changes in the temperature dependence of the KIE (ΔE_a and $A_H:A_D$) for each of the PETNR variants l-PETNR, ¹⁵N-PETNR and h-PETNR with either NADH or NADPH as coenzyme. While there is some overlap between the data due to uncertainty in both ΔE_a and $A_H:A_D$, the trend shows a progressive increase in the temperature-dependence of the KIE with increasing PETNR mass.

Previously our data have suggested, based on inferences from the temperature dependence of KIEs, that there is a significant role for fast dynamics with NADPH but potentially no role for fast dynamics with NADH in PETNR⁴². However the

trend shown in Figure 3 points to a single reaction type, where the origin(s) of the temperature dependence of the KIE is not either ‘on’ or ‘off’, but rather is a graduated continuum from l-PETNR/NADH at one extreme to h-PETNR/NADPH at the other. This finding demonstrates that the NADH and NADPH reactions are equivalent (the h-PETNR/NADH and l-PETNR/NADPH data overlap; Figure 3). Indeed, we have previously proposed that fast dynamics may not necessarily give rise to an observable temperature-dependence on the KIE in an alternative enzyme system³⁵. In the current study, we expose the subtle dependence of the H-transfer with NADH on protein motion, which is not captured using standard approaches involving measurement of the temperature dependence of the KIE. This confirms that a temperature-independent KIE does not rule out a role for (inferred) fast dynamics coupled to the reaction coordinate.

The hypothesis that the temperature-dependence of KIEs reflects vibrational coupling of enzyme chemistry to the protein is based on inference. To date, experimental efforts to test this hypothesis have generally used indirect methods to probe this coupling (e.g. enzyme variants,¹⁹⁻²¹ alternative substrates⁴² and pressure effects^{17, 27, 42}). The finding that the mass of PETNR is correlated with the observed temperature dependence of KIEs provides a direct test of the vibrational coupling hypothesis. As we show in Figure 1B and Figure S2 the only effect of making the protein ‘heavy’ is to alter the frequency of enzyme motions (according to the Born-Oppenheimer approximation), with the overall protein structure and reactive complex geometries unaffected by isotope labelling. Our observation of a continuum of temperature dependencies correlated with the increasing mass of PETNR therefore provides firm evidence that a change in the frequency of the vibrational modes of the protein alters the temperature dependence of the KIE. These data, therefore, provide direct evidence for the temperature-dependence of the KIE arising, at least in part, from vibrational coupling of chemistry to vibrational mode(s) in the protein. We note that by comparing the temperature dependence of the KIE, our data do not reflect slow motions which give rise to the reactive geometry, but only those motions which are directly coupled to the reaction coordinate.

Implications for physical models of enzymatic H-tunnelling.

Several physical models have emerged to account for the temperature dependence of KIEs. On one hand, vibronic models treat the temperature-dependence of the KIE as reflecting fast dynamics coupled to the reaction coordinate and these have been used extensively by the experimental community to analyse the temperature dependence of KIEs for enzyme catalysed H-transfer^{1, 2, 18, 20, 36, 37, 50}. Alternatively, *n*-state models describe the temperature dependence as arising from multiple reactive sub-states of the reactive enzyme-substrate conformation and not from fast motions of the protein^{2, 50}. Our data do not support the use of an *n*-state model, as it is only the frequency of protein vibrational modes that have been altered

and not the absolute reactive geometry as we show from our CT complex analysis (Figure S2).

While vibronic models of H-tunnelling are highly approximate, they have, in selected cases^{19,21}, provided some useful semi-quantitative insight. A core principle of these models is that fast protein motions coupled to the reaction coordinate – i.e. promoting vibrations – will influence the magnitude and temperature dependence of the KIE. This assumption has been highly contentious and is central to our understanding of the experimental descriptors of QMT in enzymes and physical models that describe the process. Here, we have now

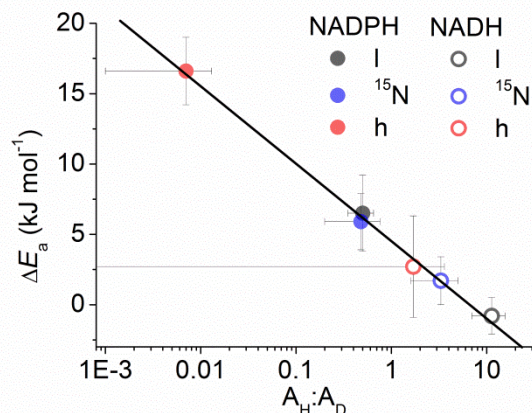


Figure 3. Correlation of extracted temperature dependence parameters from Figure 2. Solid line is a fit to a linear equation and is to aid the eye only.

established that the temperature dependence of the KIE is influenced by stable isotope labelling of the protein, providing more direct evidence for causality between fast motions and H-transfer. This establishes the importance of fast protein motions being coupled to enzyme chemistry and supports the basic conceptual ideas that have emerged from phenomenological vibronic models. However, we struggled to model the trends in the temperature dependencies of the observed KIEs on the PETNR reactions (Figure 3) using current vibronic formalisms^{20, 35, 36} without invoking unrealistic shifts in the tunnelling distance and the force constant of the apparent promoting vibration. While the frequency of harmonic vibrations shift by a factor of $\sim(\mu_i/\mu_h)^{1/2}$ (where μ is the reduced mass of the mode) – e.g. Amide I bands (peptide C=O stretch at $\sim 1650\text{ cm}^{-1}$) shift by $\sim 45\text{ cm}^{-1}$ upon ^{13}C substitution⁵¹ – the force constants ($\mu\nu^2$) of such vibrations are unperturbed by isotopic substitution. While it may be anharmonic vibration(s) (which have more complex mass dependence) in the protein that are coupled to chemistry, clearly some modification of the vibronic formalism is now required. Further, the atomistic details of tunnelling are complex and cannot be captured in simple models. This demonstrates the need to synergise experiment with computation to provide atomistic understanding of fast motions coupled to the enzyme chemistry and the origin of experimentally observed KIEs^{9, 10, 52-55}.

Conclusions

The temperature dependence of KIEs has emerged as the gold standard for experimental analysis of tunnelling in enzymes. At the simplest level, large ΔE_a and/or small $A_H:A_D$ values are thought to report on vibrational mode(s) coupled to the reaction coordinate while small ΔE_a and/or large $A_H:A_D$ values similarly arise from the absence/very weak coupling.

Experimental efforts to provide evidence for this interpretation have centred on mutational analyses, the use of alternate substrates or pressure-dependencies. Each of these methods additionally perturbs the protein in unknown ways, from altering active site and reactive complex geometry and broader protein structure to more subtle effects on the reorganisation energy, electrostatics, *etc.* Conversely, stable isotope labelling of the enzyme affects the frequency of protein (and cofactor) vibrational modes without perturbing the protein geometry or significantly altering the electrostatic environment (as the exchangeable protons are not labelled). Here we show directly that the temperature-dependence of the KIE is influenced by motion of the protein. This coupling of fast motions to H-transfer highlights the need to develop catalysis models that recognise explicitly the causal relationship between protein dynamics and enzyme chemistry.

ASSOCIATED CONTENT

Supporting Information. Data fitting parameters for CT binding titrations, observed rate constants for FMN reduction with each enzyme variant and plots of the temperature dependence of the associated KIE values. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

KIE, kinetic isotope effect; PETNR, pentaerythritol tetranitrate reductase.

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